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NEURONAL MECHANISMS OF INTELLIGENCE

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In previous work, facilitation of neuronal firing rates was observed when reinforcing transmitters and drugs were applied contingently to the cell soma after bursts of firing. This observation suggested that the activity of isolated brain cells may be susceptible to operant conditioning. An important objective of the present research was to work out the conditions that will demonstrate such neuronal operant conditioning on a reliable basis. At present, we find the most satisfactory preparation to be the brain slice, the best neurons for operant conditioning to be the large pyramidal cells in the CA1 field of dorsal hippocampus, and the most reliable reinforcing agents to be dopamine, cocaine, and the dopamine D2 receptor agonist N-0437.

There is some indication of dopamine's specificity as a reinforcer. Included among inactive substances are GABA, serotonin, acetylcholine, chlorpromazine, sulpiride, imipramine, ethanol and saline. The reinforcing actions of dopamine are blocked by chlorpromazine and the selective dopamine D2 receptor agaonist sulpiride, suggesting that dopamine's reinforcing

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19. ABSTRACT

action is mediated at dopamine D2 receptors. This conclusion is supported by positive experiments with the selective D2 receptor agonist, N-0437, which may be substituted for dopamine as a reinforcer in neuronal operant conditioning. The D2 receptor reinforcement hypothesis also is supported by a failure of the selective dopamine D1 antagonist, SCH23390, to block dopamine-reinforced operant conditioning. Preliminary results with electrical stimulation as reinforcement in brain slice experiments also indirectly supports the dopamine reinforcement hypothesis. In these experiments, mild electricl stimulation in the vicinity of dopamine terminals in the nucleus accumbens reinforced the bursting activity of accumbens cells. Noncontingent applications of the same electric stimulus failed to increase the rate of bursting.

We have begun to study the effects of delaying the presentation of reinforcement in neuronal operant conditioning. Preliminary results suggest that zero delay is optimal and that a delay as short as 0.5 sec. largely eliminates the effectiveness of the reinforcing stimulus. A steep gradient of delayed primary reinforcement also was obtained in behavioral operant conditioning (brain self-stimulation test).

Finally, we have begun to consider the biochemical events that may mediate the cellular reinforcement process. Modification of membrane proteins that control cellular firing rates is envisioned to occur only in recently-active cells primed by the influx of CA^{++} via a biochemical cascade triggered by reinforcing transmitters or drugs.

L. Stein & J.D. Belluzzi

Progress Report
AFOSR Grant #84-0325

Introduction

This research program is based on the assumption that human problem-solving behavior has evolved from the goal-seeking brain functions of lower forms. These functions in turn depend on a capacity for behavior to be strengthened or positively reinforced by its consequences, a process Skinner (1938) terms operant conditioning. A critical problem is to identify the functional brain unit whose activity is modified by the reinforcement process. Our early work suggests that the individual brain cell may serve as such a functional unit, leading us to identify the "reinforced" neuron rather than the neuronal network as the unit of goal-seeking behavior. If these assumptions are correct, it follows that the fundamental mechanisms of adaptation underlying human intelligence reside at least in part at the level of individual cells. Elucidation of the cellular mechanisms of operant conditioning may have important implications for adaptive network research.

Specific objectives of this research included: 1) demonstration that the activity of individual neurons in fact is susceptible to operant conditioning, 2) determination of the properties and limits of such neuronal operant conditioning, 3) investigation of the biochemical events that may mediate the cellular reinforcement process, and 4) comparison of the properties of neuronal and behavioral operant conditioning in order to determine important similarities and differences. Keywords: Conditioning (learning)

Methods

Brain-Slice Preparation

Rats were decapitated and their brains rapidly removed (60-90 sec) and chilled to 6°C in oxygenated artificial cerebrospinal fluid (ACSF; Dingledine, et al., 1980). Using plastic tools, the hippocampal region was rapidly dissected and rinsed repeatedly with cold ACSF to minimize cell damage. The hippocampus was positioned on a McIlwain chopper at an angle that provided parasagittal sections (15-30°) and six 400-μ slices were obtained (Tyler, 1980). The slices were individually transferred to ice-cold ACSF using a soft brush and carefully placed on the nylon mesh surface in a static chamber using an eye dropper. The slices were supported at the surface of ACSF solution in an oxygenated atmosphere (95/5 O_2/CO_2, 500 ml/min) at 35°C. At least 1 hour of incubation was allowed for recovery of physiological activity prior to the start of experiments (Schwartzkroin, 1981). Fresh ACSF was infused into the static chamber every 30-45 minute or at the end of each experiment.

Extracellular Recording and Pressure Microinjection

Single-barrel micropipette blanks (Omega Dot) were pulled and back-filled with test solution or vehicle (165 mM saline). The micropipette was connected to a pressure injector, and the tip broken back under microscopic control to

produce a droplet approximately 18μ in diameter at an injector setting of 15 p.s.i and 35 ms. Using a micropositioner, the micropipette was visually guided to targeted cells and slowly lowered until a suitable action potential was obtained. Unit activity was displayed on a digital storage oscilloscope and monitored on a loud speaker. These displays were monitored for similarity of amplitude and waveform throughout the experiment to insure that action potentials from the same cell, and only from that cell, were counted. Important criteria for the selection of suitable cells included a signal-to-noise ratio of at least 4:1 and relatively stable levels of baseline activity. Action potentials were led into an amplitude analyzer, the output of which provided digitized input to the computer. A minicomputer was programmed to count unit activity, activate the injection pump, store data on-line and analyze data off-line. A 7-channel FM recorder provided a permanent record of all essential experimental events in sequence for later analysis.

A high-pressure microinjection system was used for rapid extracellular delivery of picoliter volumes of neurotransmitters and drugs. Pressure injection is required for immediate delivery of reinforcing solutions with injection durations as short as 5 ms. High-pressure nylaflow tubing was used to connect the injection pump to the micropipette.

Single-Unit Operant Conditioning Procedures

The experimental protocol is diagrammed in Figure 1. A somewhat arbitrary decision was made in choosing which aspect of unit activity to reinforce. Since firing rates are likely to be an important vehicle for information transmission, peak rates should have high information value and might be amenable to conditioning. Thus, in initial experiments, a half-second period of relatively rapid activity was defined as the neuronal response to be reinforced (Fig. 2). These neuronal responses or "bursts" were individually determined for each unit studied. Prior to the start of conditioning, 500 successive half-second samples of neuronal activity were recorded and a frequency distribution of the number of spikes per sample was compiled. A "burst" was defined as that spike number equalled or exceeded in only 2-6 percent of the samples. During operant conditioning, reinforcements were delivered at the end of the half-second time sample containing such bursts of firing. To minimize injection artifacts, neuronal activity during and for 3 sec after each injection was excluded from analysis and had no consequences.

In later experiments, the computer program was modified to permit explicit detection of bursts of firing. In the modified program, a burst is defined as a train of firing containing n or more spikes with a maximum interspike interval of t ms: an example is shown in Figure 3 where $n = 5$ and $t = 10$ ms. Again, parameters were set for individual brain cells so that, on baseline, bursts occurred at a rate of approximately 2-6 per min. Because the new program detects the occurrence of bursts, reinforcements could be programmed to coincide precisely with the termination of bursts or to follow bursts after specified delays.

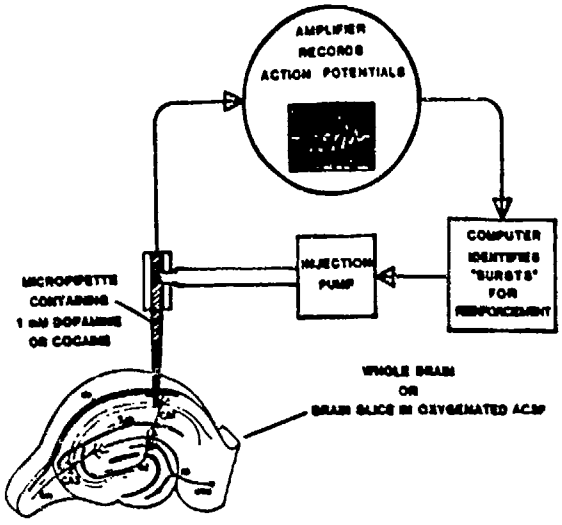

Figure 1. Protocol for operant conditioning of individual brain cells. A burst of firing of a hippocampal pyramidal cell in area CA1 activates a pressure injection pump which puffs a microinjection of dopamine or cocaine in the close vicinity of the cell soma.

The neuronal operant-conditioning method involved six stages: 1) **Baseline.** The number of "bursts" in the absence of reinforcement (operant level) was determined during a baseline period of approximately 10 minutes. 2) **Operant Conditioning.** Each "burst" was now followed by an injection of the reinforcing solution. If conditioning failed to occur after 5 minutes, the duration of the injection (and hence the dose) was increased until evidence of conditioning was obtained, or until direct pharmacological or mechanical effects interfered with recording. 3) **Extinction.** Reinforcement was terminated, and recording continued until the baseline was recovered. 4) **Matched "Free" Injections.** Noncontingent injections of the reinforcing solution were made at regular intervals to determine direct pharmacological effects on rates of firing and probability of "bursts." The pattern and number of "free" injections were matched to the pattern and number of reinforcing injections in the preceding phase of operant conditioning. The presentation of programmed free injections was delayed for 500 ms after the occurrence of "bursts" to minimize their adventitious reinforcement. (Thus, to some extent, the control involved counterconditioning rather than random presentation of reinforcement.) 5) **Washout.** A second baseline period without injections was given in order to allow residual effects of the noncontingent drug administrations to be dissipated. 6) **Reacquisition.** A second period of reinforcement was scheduled, whenever possible, in order to compare rates of original acquisition and reacquisition.

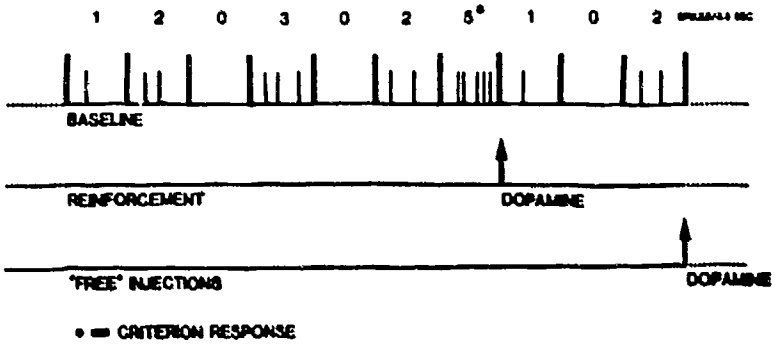

Figure 2. Diagram of procedure for defining and reinforcing neuronal responses or "bursts". Spike activity is counted and summed arbitrarily in bins of 0.5-sec duration. Prior to experiment proper, baseline recordings are made for each neuron under investigation to determine a suitable response for later reinforcement. Bins that contain *n* or more spikes are followed by reinforcement, where *n* is that number of spikes in a bin that is equalled or exceeded in 2-6 percent of all bins sampled. At the bottom, a free injection is programmed after the 10th bin, and is delivered if, as shown, the bin does not contain a burst. * = Burst.

Brain Self-Stimulation Methods

The brain self-stimulation methods have been reported previously (Black et al, 1985). Briefly, animals were implanted with bipolar electrodes and tested for brain stimulation reinforcement in a 28 x 25 x 30 cm high chamber with a lever in the rear wall. Each response delivered a 150-ms train of 0.2-ms monophasic rectangular pulses at a frequency of 100 Hz and current intensities of 75-400 μA. For initial drug testing, current intensities were individually adjusted to the lowest value that maintained stable rates of self-stimulation. Stimulus delivery and response recording (cumulative records and numerical print-outs) were under computer control.

The effects of drugs on the rewarding properties of brain stimulation also were studied in a self-stimulation test using nose-poke as the operant response. This test has been shown to be less sensitive to motor debilitating effects of drugs than tests using the lever-press response and thus provided a control for nonspecific side effects. Further analysis included measures of latency to respond and identification of extinction-like suppression patterns that indicate a receptor-mediated reward decrement process.

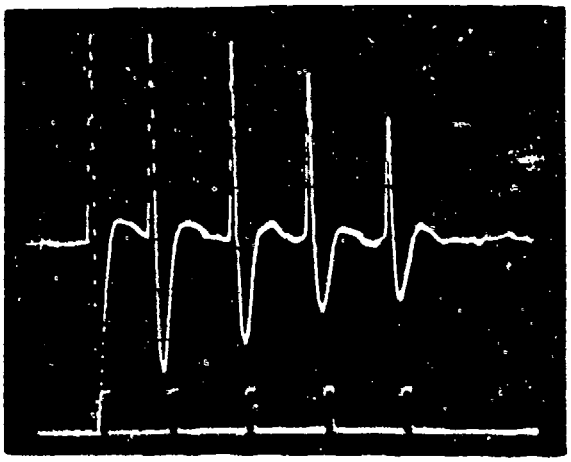

Figure 3. Burst of neuronal activity recorded from a hippocampal CA1 cell (upper trace). This pattern of firing was arbitrarily defined as a reinforceable response or "burst" and consists, for this unit, of a train of 5 or more spikes with a maximum interspike interval of 10 ms. Lower trace shows 1-ms rectangular pulses which mark each spike that is detected by an amplitude discriminator.

Conditioned Place-Preference

Animals were tested in an apparatus which consisted of two large chambers, one black plexiglas and the other white plexiglas, separated by a small central "neutral" area which was gray. The black compartment had a grid floor, wood shavings under the floor, and soap solution applied to the walls; the white compartment had mesh flooring, corn cob litter under the floor, and ethanol solution applied to the walls. Time spent in each chamber was detected by microswitches under each floor that were connected to a computer.

The conditioned place-procedure consisted of three phases. **Preconditioning (Days 1-3)**: each rat was allowed to investigate the apparatus for 15 min per day for 3 consecutive days. The time spent in each of the large compartments on the third day was used to determine the initial unconditioned preference for the two sides. **Conditioning (Days 4-11)**: each rat received 4 daily injections of the drug treatment, administered every other day. Following drug administration, the rat was confined in the less preferred environment for 30 min. Alternating with these treatments, each rat also received 4 presentations of vehicle on intervening days, and these were paired with the initially more preferred side of the apparatus. **Test (Day 12)**: no injections were administered and each rat was placed in the central area of the apparatus and the time spent in each large compartment was recorded for 15 min. The extent of place conditioning was determined by comparing time

spent in the less preferred compartment on Day 3 with time spent in the same compartment on Day 12.

Results

Evidence of Neuronal Operant Conditioning (#13, #14)[*]

Results from a representative positive experiment using dopamine as the reinforcing solution are shown for a hippocampal unit in Figure 4. In two separate periods of operant conditioning (REINF), the frequency of "bursts" and the overall firing rate were rapidly increased after approximately 5 dopamine reinforcements. The same dopamine injections administered noncontingently (MATCH) failed to increase either "burst" frequency or overall firing rate. Because neuronal activity was not increased by these noncontingent administrations, we can rule out the possibility that direct stimulant effects of dopamine caused the increases in neuronal activity that were observed in the reinforcement periods. Accordingly, we tentatively attribute these reinforcement induced increases to a neuronal process akin to operant conditioning. Note that the firing rate turned down at the end of both reinforcement periods. This effect typically is observed if high rates of bursting have been generated by the reinforcement procedure, and we tentatively attribute it to a direct inhibitory effect of dopamine when the reinforcement density (and therefore the local dopamine concentration) is excessive. In an effort to protect the unit from excessive dopamine concentrations, we typically terminate the reinforcement period at the point that the acquisition curve turns down. In the experiment shown in Figure 4, rates of bursting and overall firing continued to decline sharply after reinforcement had been terminated, suggesting rapid extinction of neuronal operant conditioning. Other units, however, sometimes respond for protracted periods in extinction (*e.g.*, see Fig. 7).

The data in the curves shown in the lower half of Figure 4 are replotted as cumulative records of bursting in Figure 5. These replots are intended to facilitate comparison with behavioral operant conditioning data (which are conventionally displayed as cumulative response curves). The neuronal data are now seen to closely resemble behavioral acquisition curves (Skinner, 1938). Two additional features of the neuronal data also are evident in the replots. First, the slope of the cumulative response curve in the second reinforcement period is somewhat sharper than that in the first period, suggesting a neuronal equivalent of enhanced reacquisition or "savings". Secondly, the response rate in the second extinction period substantially exceeded that in the first, again suggesting some persistant effect of reinforcement. Both of these features are typical of behavioral operant conditioning (see Fig. 6).

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[*] Numbers refer to project publications listed on pages 31-32.

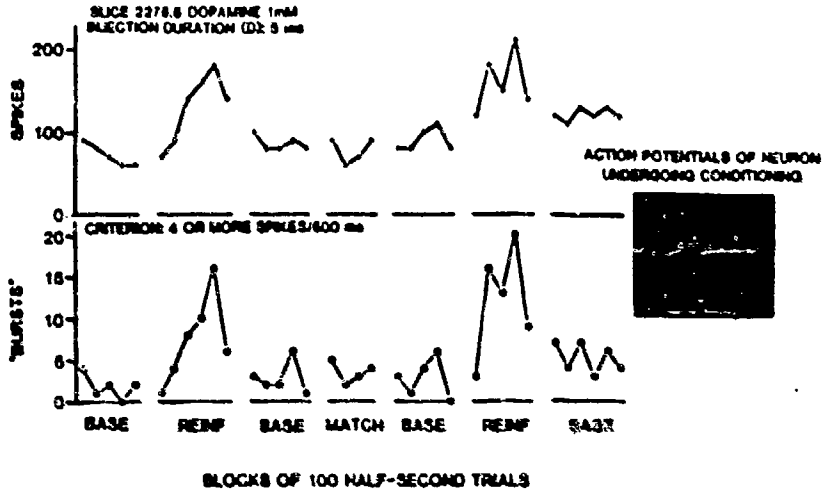

Figure 4. Operant conditioning of the activity of a CA1 pyramidal cell in a slice of dorsal hippocampus using local injections of dopamine as reinforcement. The activity of the unit throughout seven phases of a complete experiment is shown. Each point shows the number of "bursts" (lower graph) and the total number of spikes (upper graph) in successive blocks of 100 half-second samples or trials. Prior to the first baseline phase, a "burst" criterion of 4 or more spikes per half-second sample was selected. This criterion gave a "burst" rate for this unit that never exceeded 4 percent in the initial baseline period (BASE). In the reinforcement period (REINF), dopamine HCl (1 mM in 165 mM saline) was applied for 5 ms immediately after each "burst". Following a second baseline period, the same dopamine injections were delivered (MATCH) independently of the unit's behavior as a control for possible stimulant effects. The number of injections was matched to that earned during the last four periods of the reinforcement phase. "Burst" and overall spike rates were increased by the contingent dopamine injections during the reinforcement periods, but were not increased when the same injections were administered noncontingently in the matched-injection period. Inset: (upper trace) photograph of oscilloscope display of two action potentials from the unit undergoing conditioning, and (lower trace) 1-ms time markers.

Results from a positive experiment with cocaine as reinforcement are shown in Figure 7. Initially, free injections of cocaine delivered at a rate of approximately 5 per minute had no effect on the frequency of "bursts" or on the overall firing rate. In the first reinforcement period, after approximately 10 applications of cocaine, the frequency of "bursts" and the overall firing rate were sharply increased; again, both curves turned down at the end of the period, presumably because of an excessive local cocaine concentration. Unlike the experiment shown in Fig. 4, neuronal firing rates in the baseline period that followed the first phase of reinforcement did not extinguish rapidly; indeed, the peak firing rates achieved in the reinforcement phase were sustained for several minutes after the onset of extinction. Free cocaine injections ("MATCH") then were delivered at a rate of approximately 12 per minute to match the peak rate obtained in the preceding reinforcement period. These densely-packed free injections had no effect on the number of "bursts" or on the overall firing rate. In a second reinforcement period, contingent injections of cocaine again increased the frequency of "bursts" and the overall firing rate, but not to the level observed in the first reinforcement period.

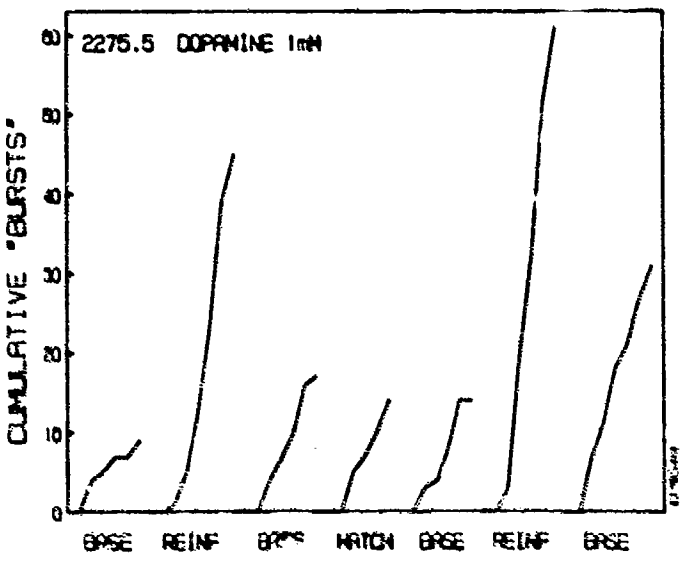

Figure 5. "Burst" data shown in lower half of Fig. 4 replotted as cumulative curves of bursting.

In control experiments, either saline was substituted for dopamine (Fig. 8) or dopamine was administered noncontingently throughout the experiment (Fig. 9). In these experiments, neither "bursts" nor overall firing rates were increased. A summary of 8 positive dopamine experiments in which it was possible to complete two reinforcement periods--as exemplified in the experiment shown in Figure 4--is shown in Figure 10. Plotted here for 8 different neurons are the mean peak rates obtained at each stage of the

experiment. Significant increases were obtained in each of the reinforcement periods when compared either to baseline control periods or to periods in which the same dopamine injections were presented independently of neuronal bursting. A similar summary of 11 positive cocaine experiments is shown in Figure 11.

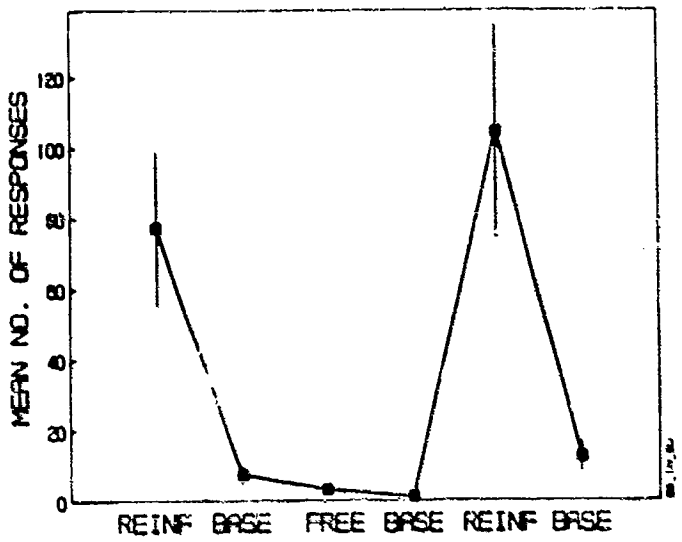

Figure 6. Results of a behavioral self-stimulation experiment which was designed to replicate the neuronal operant conditioning experiments. A nose-poke response was substituted for the burst of firing and electrical brain stimulation reinforcement was substituted for reinforcing drug injections. Experimentally naive rats, previously implanted with medial forebrain bundle electrodes, were placed in a Skinner box and trained under the same alternating contingencies used in the neuronal experiments: REINF = each nose-poke response is reinforced with a 0.15-sec train of brain stimulation; BASE = each nose-poke is recorded but has no other programmed contingencies; FREE = brain stimulations are delivered noncontingently and matched in rate to that observed in the prior reinforcement period. Note that response rate is sharply increased by reinforcement, that it declines rapidly during extinction, and that noncontingent administrations of brain stimulation do not increase nose-poking above the baseline level. Note further that response rates in the second reinforcement period exceed that in the first.

These positive results with dopamine and cocaine contrast with the negative findings of experiments in which a variety of other transmitters and drugs were surveyed (Table 1). In the columns labelled "RESULTS", the designations are as follows: ++ = evidence of operant conditioning (increased bursting in reinforcement periods and no such increase in noncontingent

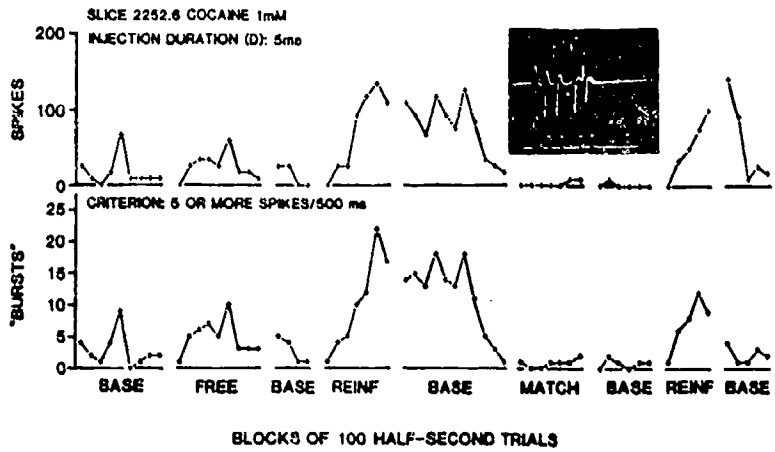

Figure 7. Operant conditioning of a pyramidal neuron in a dorsal hippocampal slice using local injections of cocaine as reinforcement. For details, see text and Fig. 4. FREE = noncontingent injections.

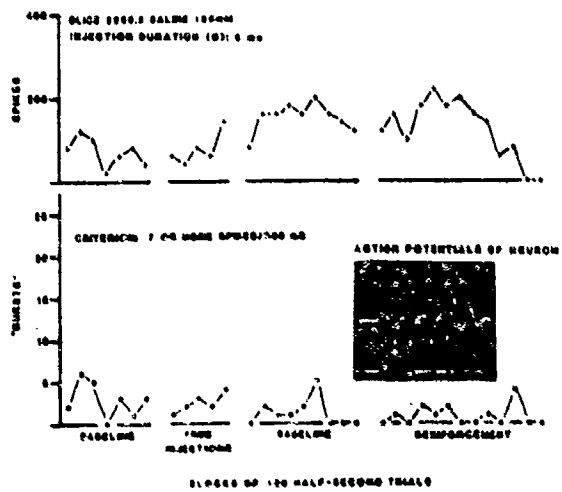

Figure 8. Saline control experiment. Failure to obtain evidence of operant conditioning of a pyramidal neuron in dorsal hippocampal slice with local injections of saline as reinforcement. For details, see text and Fig. 4.

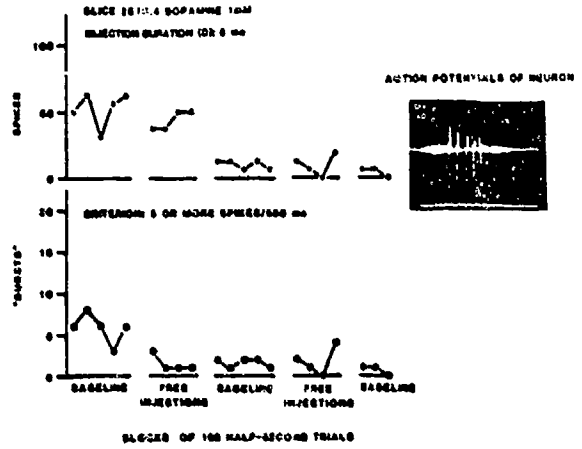

Figure 9. Control experiment with dopamine administered noncontingently to a pyramidal neuron in hippocampal slice. For details, [illegible] and Fig. 4.

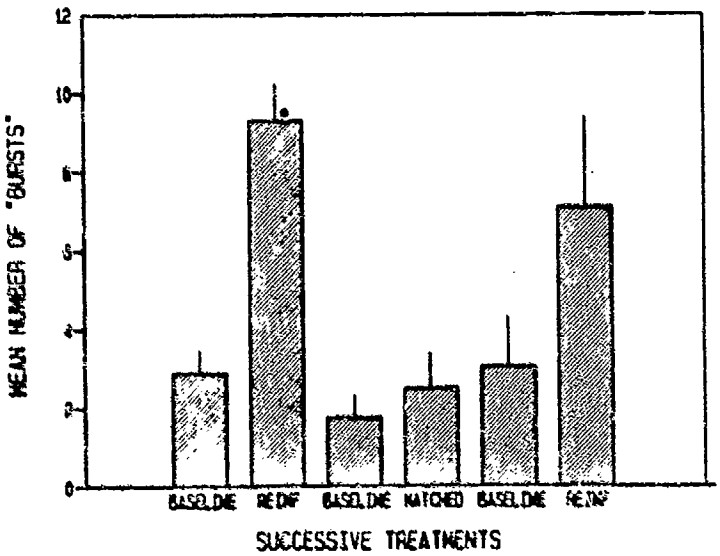

Figure 10. Summary of positive dopamine experiments. Bars show peak rates of bursting obtained in each phase of the neuronal conditioning experiment, as exemplified in Figure 4. N = 8, vertical lines represent S.E.M.s. *p < 0.05.

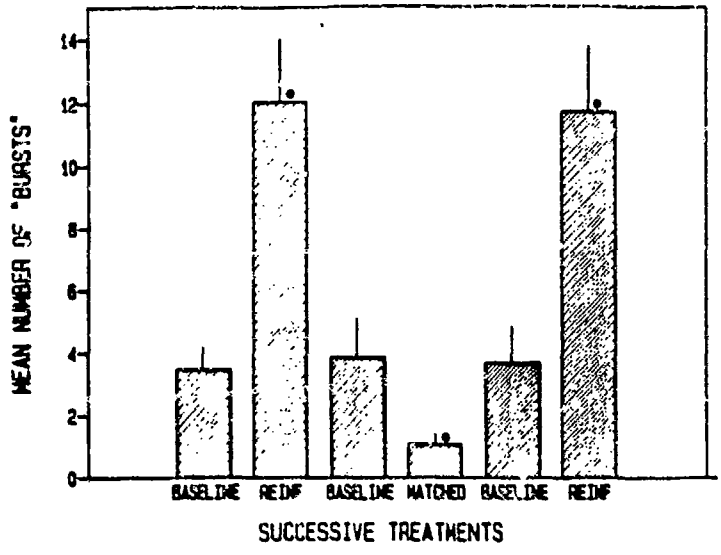

Figure 11. Summary of positive cocaine experiments. N = 11, vertical lines represent S.E.M.s. *p < 0.05. For further explanation see Figures 4 and 10.

Table 1. Summary of hippocampal brain-slice experiments.

Drug	Dose (mM)	No. of Exps.	RESULTS* ++	+	-
Cocaine	1	48	11	12	25
Cocaine (Free)	1	13	0	0	13
Dopamine	1	17	9	2	6
Dopamine (Free)	1	12	0	1	11
Norepinephrine	1	4	1	1	2
Acetylcholine	1	6	1	1	4
Serotonin	1	3	0	0	3
GABA	1	4	0	0	4
Amphetamine	1	3	0	2	1
Imipramine	1	2	0	0	2
Ethanol	1	3	0	0	3
Saline	165	5	0	0	5

*Columns are defined as follows: ++ = conditioning-like changes (increased probability of bursts following reinforcement) plus noncontingent controls, + = conditioning-like changes, but no controls, - = no evidence of conditioning. (Free) = noncontingent injections.

control periods), + = conditioning-like increases but no noncontingent controls, and - = no evidence of conditioning. The table thus indicates that 9 of the 17 dopamine experiments (or slightly more than 50%) were positive and contained noncontingent controls. In the cocaine experiments, a similar percentage of neurons exhibited increased bursting in reinforcement periods, but it was more difficult to obtain adequate noncontingent controls in the same experiments.

Evidence of Dopamine Receptor Specificity (#3, #5, #12)

Dopamine receptor antagonists were studied in neuronal operant conditioning experiments in an attempt to determine whether dopamine's reinforcing action is specifically exerted at a dopamine receptor or is due to some nonspecific action of dopamine. In initial experiments (#3), the mixed dopamine D1 and D2 receptor antagonist chlorpromazine completely blocked dopamine's reinforcing action in neuronal operant conditioning (Fig. 12). In these experiments, hippocampal units reinforced with dopamine (DA-REINF) again exhibited significantly higher bursting rates than control neurons reinforced with saline (SAL-REINF). When chlorpromazine was added to the dopamine solution (DA + CPZ), the reinforcing action of dopamine was abolished; indeed, the dopamine-chlorpromazine mixture apparently suppressed the rate of bursting below the saline control and below those neurons that had received chlorpromazine alone (CPZ) as reinforcement.

The availability of new drugs with greater selectivity than chlorpromazine has enabled us to distinguish between effects exerted at dopamine D1 and D2 receptors (Fig. 13). When the selective D2 antagonist, sulpiride, was added to dopamine (DA + SUL), the reinforcing action of dopamine was abolished and the rate of bursts was suppressed to the saline control level. On the other hand, when the dopamine D1 receptor antagonist, SCH 23390, was mixed with dopamine (DA + SCH), the reinforcing action of dopamine was unaffected or possibly even slightly increased. These results suggest that dopamine's reinforcing effects are exerted at dopamine D2 receptors. This conclusion is supported by positive experiments with the D2 receptor agonist, N-0437, which may be substituted for dopamine as an effective reinforcer in neuronal operant conditioning (Fig. 14). Although higher concentrations of N-0437 than dopamine were required for neuronal operant conditioning, it is our impression that at these higher concentrations N-0437 is a more reliable reinforcing agent.

Preliminary results with electrical stimulation as reinforcement in brain slice experiments also indirectly supports the dopamine reinforcement hypothesis. In these experiments, mild electrical stimulation delivered directly to a localized site in the brain slice was substituted for the reinforcing dopamine injections in a typical neuronal operant conditioning procedure. The parameters of electrical stimulation were identical to those used in behavioral self-stimulation studies. In nucleus accumbens brain slices, mild electrical stimulation in the presumed vicinity of the dopamine projections reinforced the bursting of accumbens cells (Fig. 15). Noncontingent applications of the same

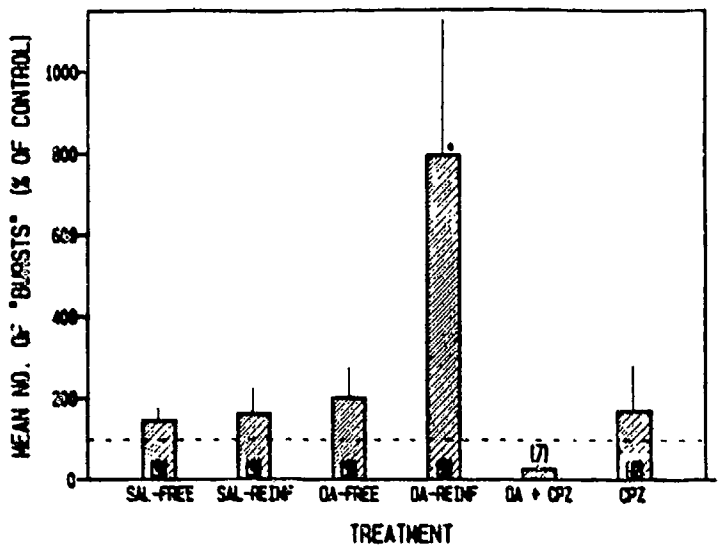

Figure 12. Chlorpromazine blocks operant conditioning of individual CA1 cellular activity in slices of hippocampus, using local applications of dopamine as reinforcement (see Methods and Fig. 4 for procedure). Neurons reinforced with 1-mM dopamine (DA-REINF) exhibited significantly more "bursts" than controls reinforced with saline (SAL-REINF). When 1-mM chlorpromazine was added to the dopamine solution (DA + CPZ), the reinforcing action of dopamine was abolished and the rate of "bursts" was suppressed below the saline control. Neurons that received chlorpromazine alone (CPZ) exhibited the same number of "bursts" as those that had received saline. SAL-FREE = noncontingent saline injections; DA-FREE = noncontingent dopamine injections.

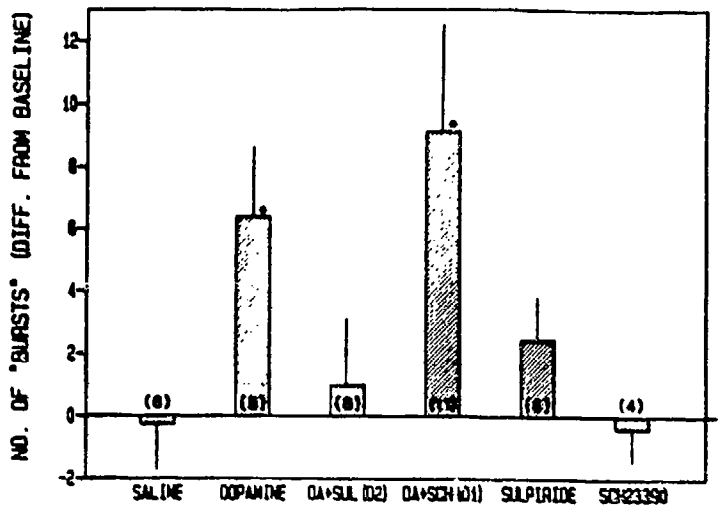

Figure 13. Sulpiride, but not SCH 23390, blocks operant conditioning of individual CA1 cellular activity in slices of hippocampus, using applications of dopamine as reinforcement (see Methods and Fig. 4 for procedure). Neurons reinforced with 1-mM dopamine (DOPAMINE) exhibited significantly more bursts than controls reinforced with saline (SALINE). When sulpiride (10 mM) was added to the dopamine solution (DA + SUL), the reinforcing action of dopamine was abolished and the rate of bursts was suppressed to the saline control level. On the other hand, when 1-mM SCH23390 was added to the dopamine solution (DA + SCH) the reinforcing action of dopamine was unaffected.

stimulation failed to increase the rate of bursting. In hippocampal slices, however, similar electrical stimulation experiments produced no evidence of operant conditioning. In this case (Fig. 14), contingent and noncontingent electrical stimulation produced similar and much smaller changes in the rates of bursting. It is possible that the positive results in nucleus accumbens may be associated with the heavy density of dopamine projections to this region, while the negative results in hippocampus may be associated with its much thinner dopamine innervation.

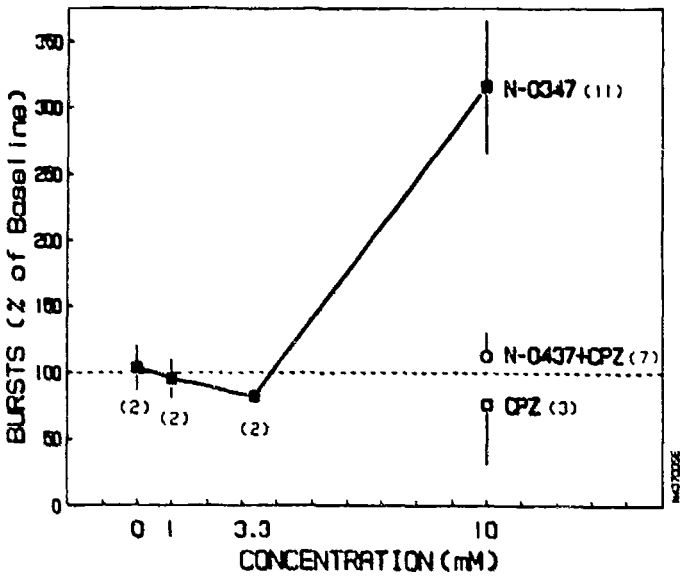

Figure 14. Neuronal operant conditioning obtained with N-0437 reinforcement as a function of drug concentration. The reinforcing action of N-0437 (10 mM) was abolished by chlorpromazine (1 mM).

Effects of Delayed Reinforcement in Neuronal Operant Conditioning (#2)

In behavioral operant conditioning, it is well established that the effectiveness of the reinforcement is sharply reduced when the presentation of the reinforcing stimulus is substantially delayed after the correct response (Renner, 1964). The brain self-stimulation method, by eliminating the necessity for consumatory responses, permits precise temporal control of the interval between the operant response and primary reinforcement. Using this method, we found that delays even as short as one second markedly impede the acquisition of self-stimulation behavior (Fig. 16). Demonstration of a similar delay-of-reinforcement decrement in neuronal operant conditioning experiments would provide strong support for the hypothesis that cellular reinforcement processes underlie behavioral reinforcement.

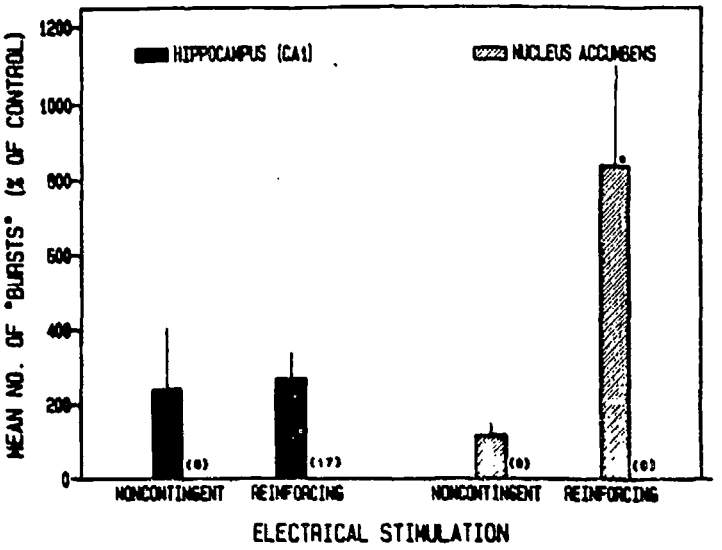

Figure 15. Neuronal operant conditioning experiments with electrical stimulation as reinforcement in hippocampal and nucleus accumbens brain slices. The electric stimulus (50-100 μA, 100 Hz, 100 ms in duration) was delivered to a localized site in the brain slice within approximately 1 mm of the recording micropipette, either contingently after bursts of firing (REINFORCING), or independently of neuronal activity (NONCONTINGENT). Bars show peak rates of bursting obtained with each procedure as a percent of baseline. In nucleus accumbens, very large increases in bursting were obtained with reinforcing stimulation; these increases are suggestive of operant conditioning since noncontingent stimulation was ineffective. In hippocampus, on the other hand, there was no evidence of operant conditioning since reinforcing and noncontingent stimulation produced equal (and much smaller) increases in bursting.

Because N-0437 produces highly reliable baselines of operant conditioning, this compound was used as the reinforcing substance in our initial work on the delay of reinforcement problem. A representative experiment comparing the efficacy of immediate and delayed reinforcement is shown in Figure 17. Immediate and delayed reinforcement procedures were identical, except that the delay procedure interposed an interval of 500 ms between the last spike in the burst and the presentation of reinforcement period (DELAYED REINF). After causing a brief increase in the bursting rate, delayed reinforcement had no sustained effect or perhaps even suppressed the rate of bursting. On the other hand, in a subsequent period of immediate reinforcement (IMMEDIATE REINF), bursting rates increased sharply in a characteristic acquisition curve. A similar result is shown for a second unit in an experiment in which the sequence of immediate and delayed reinforcement was reversed (Fig. 18). The efficacy of operant conditioning associated with reinforcement delays of 0, 100,

200, or 500 ms was determined in an experiment involving 32 units; each unit received operant conditioning at a single reinforcement delay. A delay-of-reinforcement gradient was generated by averaging the peak bursting rates at each delay (Figure 19). The curve indicates that reinforcement delays exceeding 200 ms largely eliminate the effectiveness of N-0437 reinforcement in CA1 operant conditioning. Such a steep gradient of reinforcement delay is consistent with that obtained in behavioral experiments, and supports the idea that the neuronal operant conditioning process may underlie the behavioral operant conditioning process.

Possible Role of Norepinephrine in Neuronal Operant Conditioning

Because of the important role of norepinephrine as a first messenger in the phosphoinositide sequence, we reexamined the efficacy of norepinephrine as a reinforcing substance in neuronal operant conditioning. Norepinephrine's triggering action in phosphoinositide is exerted exclusively at α_1-noradrenergic receptors; it therefore seemed logical to retest norepinephrine in a mixture containing the β-noradrenergic receptor antagonist, propranolol, in an attempt to produce a relatively pure α-noradrenergic receptor activation. Initial data presented in Figure 20 in fact suggest that selective activation of α-noradrenergic (NE + PROP) receptors may provide more effective reinforcement than simultaneous activation of α- and β-noradrenergic receptors

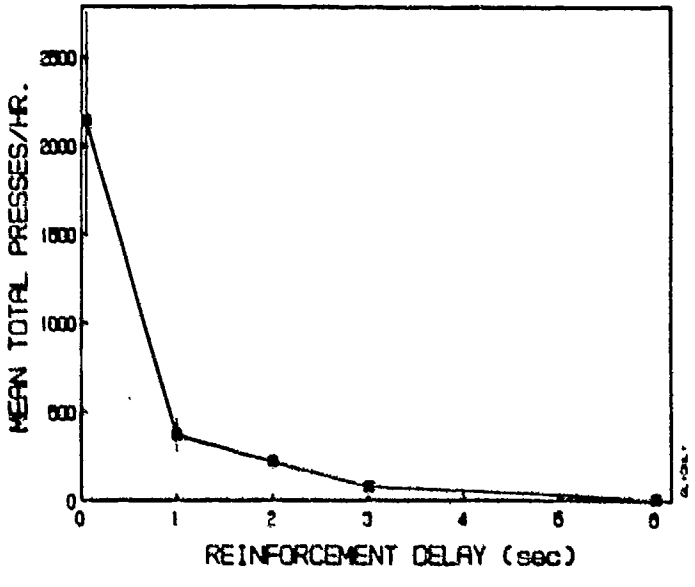

Figure 16. Acquisition of operant behavior (hypothalamic self-stimulation) as a function of reinforcement delay. Total lever-press responses on Day 1 of training are shown for different groups of animals reinforced after the indicated delay. Note that a delay of only 1 sec produced a rate decrease of approximately 90%. Bars represent ± S.E.M.

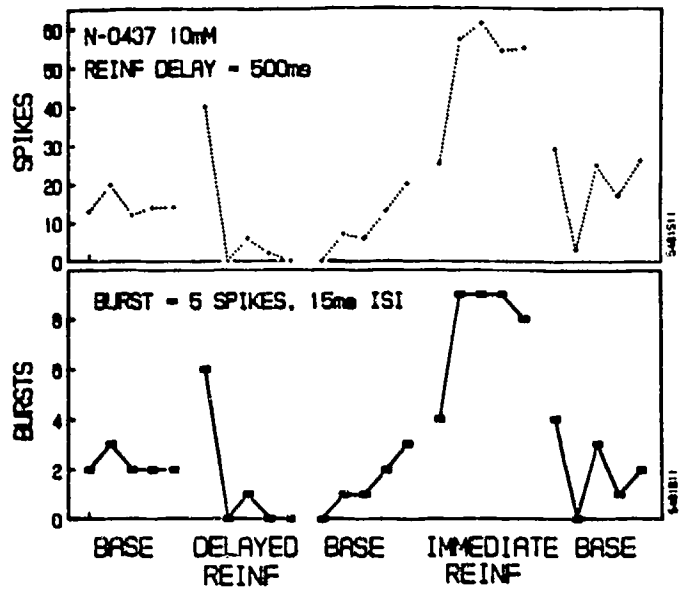

Figure 17. A representative neuronal operant conditioning experiment in which the efficacy of immediate and delayed (500 ms) reinforcement are compared. The delayed reinforcement procedure (DELAYED REINF) produced a brief, but unsustained, increase in bursting; on the other hand, immediate reinforcement (IMMEDIATE REINF) produced a characteristic acquisition curve.

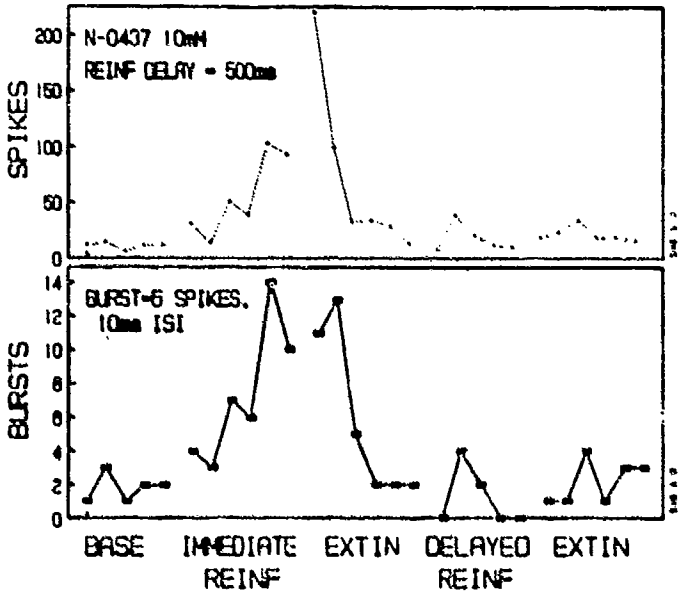

Figure 18. A second example of a neuron for which a reinforcement delay of 500 ms (DELAYED REINF) eliminated the reinforcing action of N-0437. Compare with Figure 17.

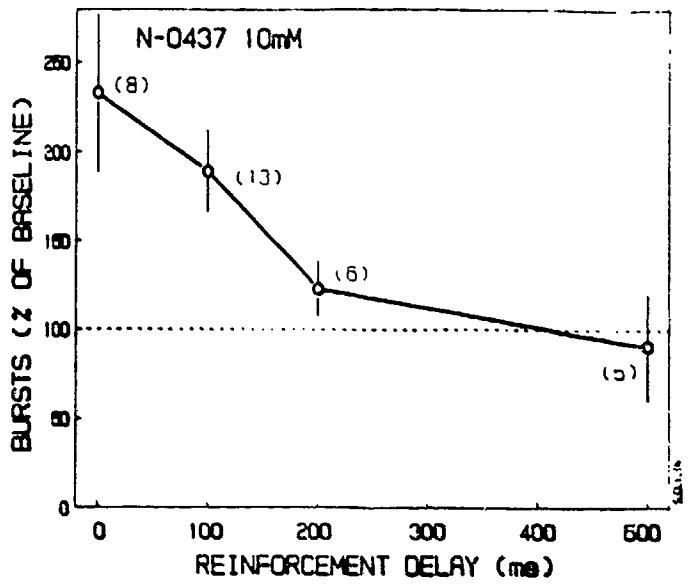

Figure 19. Delay of reinforcement gradient in neuronal operant conditioning with N-0437 (10 mM) as reinforcement. Number of neurons tested at each reinforcement delay indicated in parentheses. Vertical lines represent ± S.E.M.s.

together (NE). Preliminary experiments also suggest that norepinephrine may be combined with otherwise ineffective doses of N-0437 to produce neuronal operant conditioning (Fig. 21).

Operant Conditioning of Single Units in Whole Brain (#6)

Whole brain preparations have been used to identify target cells, in addition to hippocampal CA1 neurons, that may be suitable for operant conditioning. In these experiments, electrical stimulation of the medial forebrain bundle (delivered through conventional, permanently implanted electrodes whose reinforcing efficacy had previously been demonstrated in behavioral self-stimulation tests) provided reinforcement for neuronal operant conditioning. The rats were anesthetized with urethane (1.2 g/kg, I.P.), and an extracellular recording electrode was progressively lowered from the surface of the cortex through the nucleus accumbens (a major target for the dopamine fibers in the MFB). Neurons that exhibited operant conditioning were found exclusively in medial frontal cortex (Fig. 22).

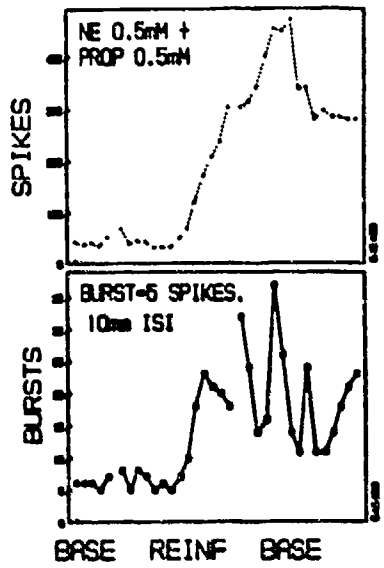

Figure 20. Neuronal operant conditioning produced by combined administration of norepinephrine (NE 0.5 mM) and the β-noradrenergic receptor antagonist propranolol (PROP 0.5 mM). Prolonged elevation of firing rates after reinforcement is discontinued is characteristic of this combination of drugs.

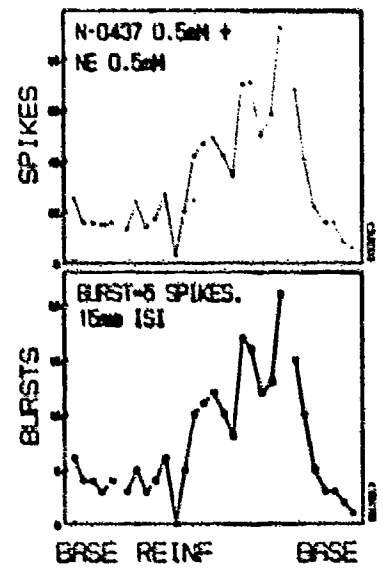

Figure 21. Neuronal operant conditioning produced by combined administration of norepinephrine (NE 0.5 mM) and the D2 dopamine receptor agonist N-0437 (0.5 mM). This mixture of drugs sometimes produces direct stimulant effects on neuronal firing rates (not shown).

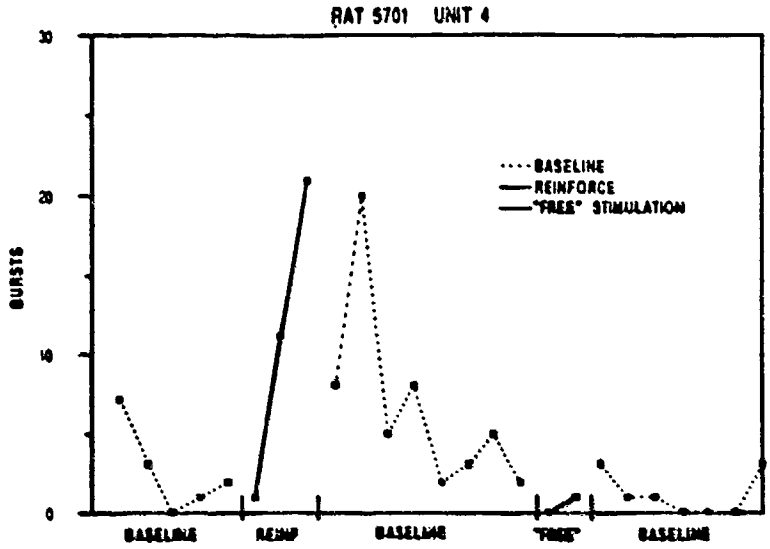

Figure 22. Operant conditioning of a single frontal cortical unit in the intact brain of an anesthetized rat using electrical stimulation (150 msec train of 0.2 msec pulses at 100 Hz, 400μA) of the medial forebrain bundle through an implanted electrode as reinforcement. In this experiment, a sharp acquisition curve was produced by contingent presentations (REINF) of the rewarding electrical stimulus after bursts of firing; noncontingent presentations ("FREE") of the same stimulus were ineffective. BURST = train of 6 or more spikes with a maximum interspike interval of 15 ms.

Hippocampal Self-Stimulation (#7)

The success of our neuronal operant conditioning experiments in hippocampal brain slices led us to reexamine hippocampal self-stimulation at the behavioral level. Although there are published reports that rats will lever-press for electrical stimulation of the dentate gyrus or other hippocampal sites, the rates of such hippocampal self-stimulation are very low (Ursin, Ursin and Olds, 1966). In an initial experiment, we were unable to train rats to bar-press for hippocampal self-stimulation, even after extensive shaping; however, the a nose-poke response for the hippocampal reward was rapidly learned (Fig. 23). In a second experiment, naive rats with electrodes in the CA1, CA3, or dentate gyrus areas of hippocampus were trained to work for brain stimulation in the nose-poke test, then were switched to a bar-press test for five sessions, and finally were returned to the nose-poke test. When the rats were switched to the bar-press test their self-stimulation rates abruptly fell to 20% of the nose-poke rate; the depressed rates recovered immediately when the rats were returned to the nose-poke task. In pharmacological experiments, we found that amphetamine (1 mg/kg) dramatically increased

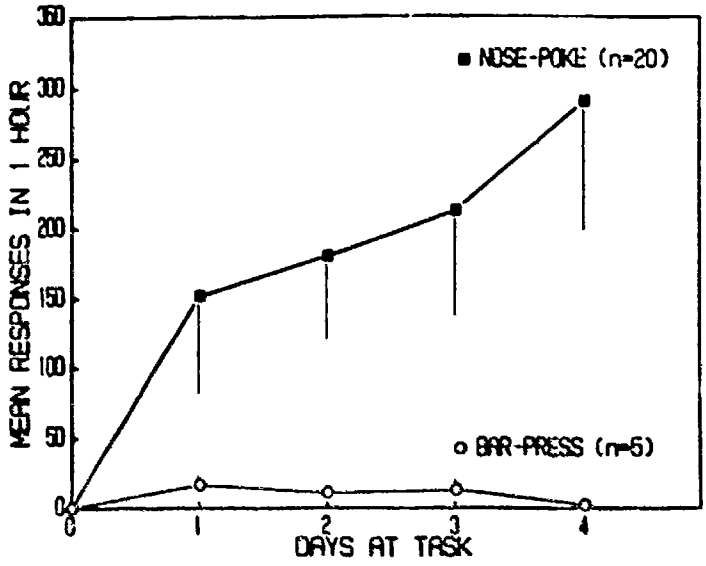

Figure 23. Acquisition curves of hippocampal self-stimulation for two groups of rats reinforced either for nose-poke or bar-press responses. Rats learned the nose-poke response spontaneously, but could not learn to press a bar for the hippocampal stimulation, even with extensive shaping. Bars represent ± S.E.M.s.

nose-poke self-stimulation rates at all 3 brain sites. Self-stimulation rates were increased as much as 10-fold in some cases, strongly implicating a catecholamine in hippocampal reward. Naloxone (2 mg/kg) selectively decreased self-stimulation at the CA3 site, suggesting that reinforcement associated with this site may be regulated by endogenous opioids.

Nucleus Accumbens Self-Stimulation: Evidence of Endorphin-Mediated Reinforcement (#2, #8, #19, #20, #21)

The opiate antagonist naloxone suppresses self-stimulation of the nucleus accumbens and other brain areas rich in endorphins. In a series of experiments, we showed that the suppressive of naloxone is independent of response effort (#21), centrally mediated (#2, 20) and resembles the effects of nonreinforcement or extinction in its time course (#19). These results support the hypothesis that nucleus accumbens self-stimulation depends upon the activation of endorphin neurons and the consequent release of endogenous opioids which function as reward transmitters. If this hypothesis were correct, enhancement of endorphin release would decrease the behavioral efficacy of naloxone due to increased competition for reward receptors. To test this idea (#8), endorphin release was varied by systematic manipulation of the pulse frequency of the rewarding electrical stimulus. Animals with nucleus accumbens electrodes were trained in one-hour daily sessions to nose-poke for

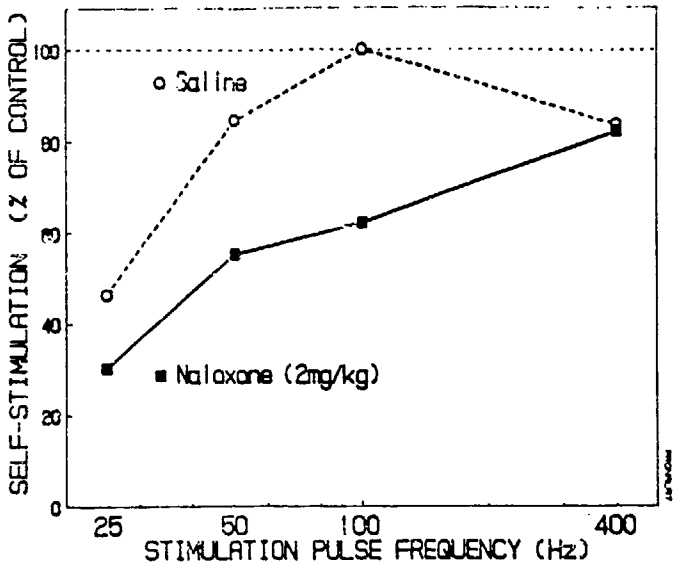

Figure 24. Naloxone suppression of nucleus accumbens self-stimulation varies inversely with stimulation pulse frequency. Mean self-stimulation rates in the last 45 minutes of the 1-hr test are plotted as a function of stimulation frequency (N = 9 at each point). Naloxone scores are expressed as mean percent of the saline control at the same pulse frequency. Saline scores are the mean percent of the saline rate at 100 Hz.

electrical brain stimulation (150-msec train of 0.2 msec monophasic square pulses, 100 Hz, 375 μA). After self-stimulation rates had stabilized, baseline pulse frequency-response curves were established for each animal in the range, 25-400 Hz. Such pulse frequency-response curves were then established following injections of naloxone (2 mg/kg, s.c.) and saline (1 ml/kg, s.c.). The open circles in Figure 24 show saline self-stimulation rates at each pulse frequency as a percent of the saline rate at 100 Hz (the standard pulse frequency used throughout training). Black squares represent naloxone self-stimulation scores as a percent of saline self-stimulation scores at each of the indicated pulse frequencies. Consistent with the endorphin reward hypothesis, naloxone suppression of self-stimulation decreased substantially with increasing pulse frequency. These results are consistent with the idea that nucleus accumbens self-stimulation depends on the activation of endorphin reward receptors and that naloxone's suppressant action is associated with the blockade of these receptors.

Conditioned Place-Preference: Evidence of Dopamine D2 Receptor Involvement in Behavioral Reinforcement (#9)

The neuronal operant conditioning experiments implicate a dopamine D2 receptor in reinforcement processes. This hypothesis was tested in a behavioral experiment, in which the conditioned place-preference method was used to measure reinforcement. Previous work has established that injections of reinforcing drugs in one compartment of a 2-compartment apparatus induce a preference for the compartment in which the reinforcing injections had been made. Dopamine D1 and D2 receptor agonists were tested for their reinforcing action in this test. N-0437 (3 mg/kg), a dopamine D2 receptor agonist, induced a significant place preference, whereas SKF 38393 (20 mg/kg), a specific D1 receptor agonist, induced no such preference (Fig. 25). These results are consistent with those of the neuronal operant conditioning experiments in suggesting that the D2, and not the D1, receptor is associated with reinforcement.

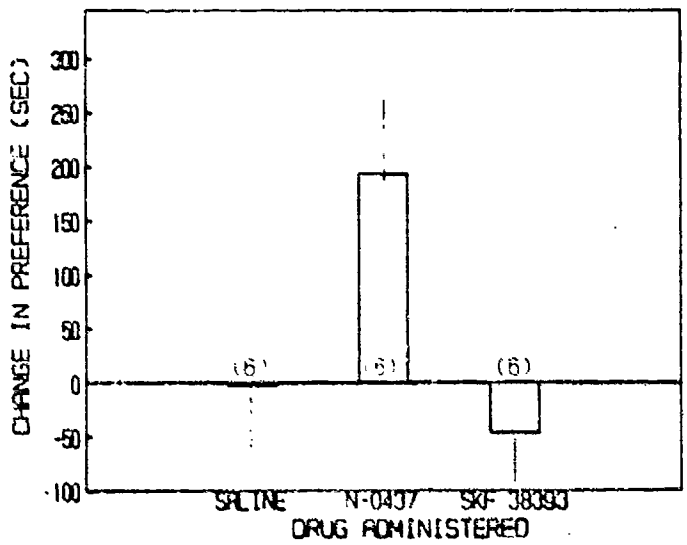

Figure 25. Conditioned place preference induced by the dopamine D2 receptor agonist N-0437 (3 mg/kg). The dopamine D1 receptor agonist, SKF 38393 (20 mg/kg), had no significant effect. Bars represent ± S.E.M.s.

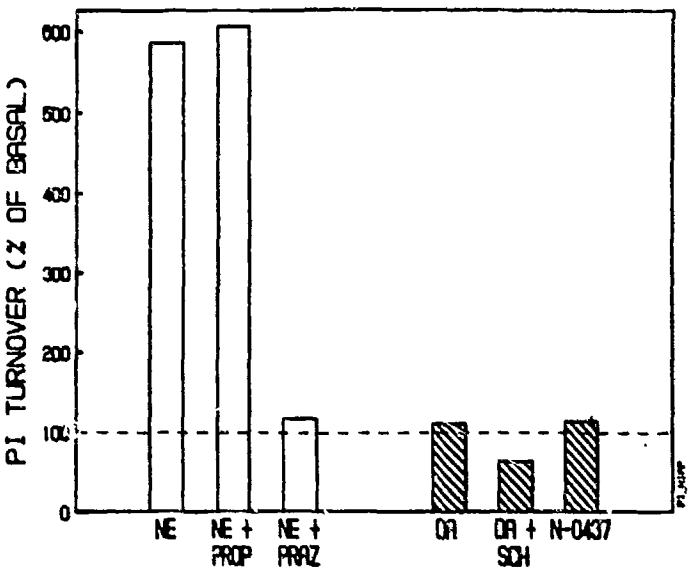

Figure 26. Phosphoinositide (PI) turnover in hippocampal brain slices induced by catecholamine receptor activation. Norepinephrine (NE) produced a significant increase in PI turnover that was blocked by the α-receptor antagonist prazocine (PRAZ), but not by β-receptor antagonist propranolol (PROP), confirming that PI turnover is induced by α-noradrenergic receptor activation. Dopamine and the dopamine D2 receptor agonist N-0437 had no effect on PI turnover, and a mixture of dopamine and the dopamine D1 receptor antagonist SCH23390 (SCH) even seemed to suppress PI turnover.

Biochemical Experiments

Involvement of the dopamine D2 receptor both in neuronal and behavioral reinforcement raises the question of which second messenger may mediate its intracellular effects. The dopamine D2 receptor, unlike the D1 subtype, is not linked to adenylate cyclase; this excludes cyclic AMP as a second messenger. Although dopamine is not thought to be a potential first messenger in the inositide pathway, the possibility that D2 receptor activation can stimulate this pathway has not been experimentally excluded. Accordingly, we used the method of Berridge et al (Berridge, Downes & Hanley, 1982) to monitor activation of the inositol pathway *in vitro* by exposure of tissue to various agonists.

Results from experiments using hippocampus are summarized in Figure 26. Norepinephrine (0.1 mM NE) stimulates PI turnover as been reported previously (Berridge et al 1982). This effect is blocked by the α-noradrenergic receptor

antagonist, prazocine (NE + PRAZ), but not by the β-noradrenergic receptor antagonist, propranolol (NE + PROP). No stimulation of PI turnover was observed after treatment with dopamine (1mM DA), the dopamine D2 receptor agonist, N-0437, or a combination of dopamine and the dopamine D1 receptor antagonist, SCH 23390 (DA + SCH). These results suggest that dopamine D2 receptor activation does not trigger the formation of phosphoinositide second messengers.

Conclusions

Cellular applications of dopamine or cocaine to spontaneously active CA1 pyramidal cells in slices of rat hippocampus had opposite effects on subsequent firing rates, depending on the activity pattern of the neuron at the time of drug administration. If the neuron had been firing rapidly just before the injections, the firing rate was increased. However, if the neuron had been firing slowly or was silent at the time of injection, the firing rate was unaffected or decreased. In other words, the action of locally-applied dopamine or cocaine on hippocampal cells was activity-related in a way that formally resembles the action of conventional reinforcers on behavior. A food pellet delivered immediately after a lever-press response increases lever pressing, whereas the same pellet delivered independently of the lever-press response has no effect or even may suppress the behavior. These observations, therefore, are consistent with the possibility that the activity of individual neurons may be operantly conditioned by direct cellular applications of reinforcing transmitters or drugs. If so, and since it is unlikely that a brain cell would display a gratuitous capacity for operant conditioning, the individual neuron could be an important functional unit for positive reinforcement in the brain.

These conclusions are supported by preliminary results on the effects of delay of reinforcement in the neuronal operant conditioning paradigm. In behavioral operant conditioning, it is well established that the effectiveness of reinforcement is sharply reduced when the presentation of the reinforcing stimulus is substantially delayed; indeed, we found that a delay as short as one second caused a severe decrement in the acquisition of self-stimulation behavior. A similar delay-of-reinforcement decrement was observed in the neuronal operant conditioning experiments; in this case, however, delays as short as 200 ms largely eliminated the facilitating action of N-0437 on hippocampal CA1 bursting activity. The steep gradient of effectiveness of delayed reinforcement makes it unlikely that nonspecific stimulation or some artifact of the injection procedure accounts for the increase in neuronal firing. Rather, the stringent requirement for contingency supports the idea that we have identified a neuronal conditioning process that may be closely related to behavioral operant conditioning.

We have begun to work out the conditions that will demonstrate neuronal operant conditioning on a reliable basis. Thus, we find at present the most

satisfactory preparation for our operant conditioning experiments to be the brain slice, the best neuron to be the large pyramidal cells in the CA1 field of dorsal hippocampus, the most appropriate neuronal response for reinforcement to be a burst of activity containing 3 or more spikes, and the most reliable reinforcing agents to be dopamine, cocaine, and a newly developed and selective dopamine D2 receptor agonist, N-0437. There is already an indication of specificity in the role of the dopamine receptor in cellular reinforcement. Included among substances that are ineffective are GABA, serotonin, acetylcholine, imipramine, ethanol, and saline. The reinforcing action of dopamine is blocked by chlorpromazine and the selective dopamine D2 antagonist sulpiride, suggesting that dopamine's cellular reinforcing action is mediated at D2, rather than D1, receptors. As noted above, this conclusion is supported by positive experiments with the selective D2 receptor agonist, N-0437, which may be more reliable than dopamine as a reinforcer in neuronal operant conditioning. The D2 receptor reinforcement hypothesis is also supported by a failure of the selective dopamine D1 antagonist, SCH 23390, to block dopamine-reinforced operant conditioning. In fact, the combination of dopamine and SCH 23390 provides slightly more reliable operant conditioning than dopamine alone, suggesting that selective activation of dopamine D2 receptors may provide greater reinforcement than simultaneous activation of D1 and D2 receptors together. Preliminary results with electrical stimulation as reinforcement in brain slice experiments also indirectly supports the dopamine reinforcement hypothesis. In these experiments, mild electric stimulation in the vicinity of dopamine axons in the nucleus accumbens reinforced the bursting of accumbens cells. Noncontingent applications of the same electric stimulation failed to increase the rate of bursting.

Dopamine seems to be a more effective reinforcer than norepinephrine; however, some recent experiments suggest that the efficacy of norepinephrine is increased if it is combined with the β-noradrenergic receptor antagonist, propranolol. This result suggests that selective activation of α-noradrenergic receptors may provide greater reinforcement than simultaneous activation of α and β receptors together, just as selective activation of dopamine D2 receptors may be more favorable than the joint activation of D1 and D2 receptors.

A troublesome feature of the present experiments is the fact that relatively high concentrations of the effective agents (1 mM of dopamine and cocaine, and 10 mM of N-0437) were required for reinforcement. However, it should be clear that total drug dose is determined not only by the concentration of the solution injected, but also by other injection parameters, such as duration and volume. Because drug injections in this experiment had to be delivered to individual cells in close contingency to bursts of activity, it was necessary to use exceedingly short injection durations (5-20 ms) and small volumes (0.5-3 picoliters). After diffusion to action sites, these minute droplets of drug presumably are diluted to concentrations comparable to those produced in more conventional neuropharmacological studies, where lower initial concentrations of drug are applied in greater volumes and for much longer durations. In any case, until more is known about the local distribution

and metabolism of the reinforcing agents, our strategy has been to determine effective concentrations empirically and to compare these relatively high reinforcing concentrations with identical control injections applied noncontingently or after a delay.

Finally, we have begun to consider the biochemical events that may mediate the cellular reinforcement process. What is required is a mechanism that will satisfy the following conditions: 1) if a brain cell with the capacity for positive reinforcement discharges in a burst of activity, and 2) if that cell's catecholamine or endorphin "reinforcement" receptors are activated shortly thereafter, then and only then, 3) will membrane proteins, which control the cell's excitability, be modified to increase the probability of future firing. Clearly, only recently active cells can be eligible for reinforcement. Three possible ionic markers of recent activity, and hence reinforcement eligibility, are Na^+ or Ca^{++} influx, or K^+ efflux. Since calcium influx is a universal signal for the activation of intracellular biochemistry, we assume that calcium influx may be the ionic signal that primes the cell for the reinforcement message.

The next step is to identify the intracellular event or second messenger that may be activated by the reinforcing signal. Such second messengers could include cyclic AMP, or the phosphoinositide second messengers, diacylglycerol and inositol triphosphate, or other substances, including some that are still unknown. Following the work of Kandel (1984), we (Stein and Belluzzi, 1986) speculated initially that the second messenger associated with the reinforcement signal might be cyclic AMP, in part because the existence of a dopamine-activated adenylate cyclase is well established (Greengard, 1978). However, such dopamine activation of adenylate cyclase is known to be mediated via dopamine D1 receptors, while our work suggests that a D2 receptor is more likely to be involved in cellular reinforcement (Belluzzi and Stein, 1986). Furthermore, it is well established that enkephalins and rewarding opiate drugs inhibit, rather than activate, adenylate cyclase, and it has been speculated that such inhibition is involved in their reinforcing action. It seems probable, therefore, that second messengers other than cAMP are involved in the biochemical mechanism of positive reinforcement. Unfortunately, the second messengers associated with dopamine D2 receptor activation are presently unknown; our own work, described above, demonstrates that phosphoinositide second messengers are not involved. Nevertheless, diacylglycerol and inositol triphosphate continue to intrigue us, in part because their α-noradrenergic first messenger receptor has been implicated in behavioral reinforcement and long-term memory (Stein, 1978), and in part because their associated third messenger, protein kinase C, is a calcium-dependent brain kinase, which can be activated to modify membrane proteins that control cellular excitability (Nishizuka, 1986). It is also known that calcium influx shifts protein kinase C from the cytosol to its membrane bound form (Schulman, 1984), thereby priming and positioning the enzyme for activation by an extracellular (reinforcing?) signal. Finally, inositol triphosphate, by mobilizing intracellular calcium, could activate the gene

transcription and protein synthesis that may be necessary for the long-term behavioral changes induced by positive reinforcement.

In brief, the proposed mechanism of positive reinforcement is envisioned to operate in the following manner. In certain cells capable of operant conditioning, a burst of firing leading to strong Ca^{++} influx induces a state of reinforcement eligibility by briefly shifting protein kinase C to its membrane bound form. In this window of opportunity, protein kinase C is oriented in close conjunction to first-messenger reinforcement receptors and their second-messenger enzyme, phospholipase C. At this point, activation of the first-messenger reinforcement receptors by norepinephrine or other appropriate transmitters stimulates the formation of diacylglycerol and inositol triphosphate. These second messengers, in turn, activate the membrane-bound protein kinase C for short-term modification of membrane proteins that control cellular excitability. Long-term changes in excitability may be initiated by the same intracellular messengers, which could switch on genomic events leading to long-term behavioral changes.

Project Publications.

1. Black, J. Cellular Aspects of Operant Conditioning. Dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Pharmacology and Toxicology, 1987.
2. Black, J., Belluzzi, J.D. & Stein, L. Reinforcement delay of one second severely impairs acquisition of brain self-stimulation. *Brain Research*, 359:113-119, 1985.
3. Belluzzi, J.B., Trujillo, K.A., Sun, C.D. & Stein, L. Naloxone suppresses self-stimulation by central blockade of reinforcement. *Society for Neuroscience Abstracts*, 11:1172, 1985.
4. Stein, L. & Belluzzi, J.D. Operant conditioning of hippocampal neurons: chlorpromazine blocks reinforcing actions of dopamine. *Society for Neuroscience Abstracts*, 11:873, 1985.
5. Trujillo, K.A., Tabrizi, P.R., Belluzzi, J.D. & Stein, L. Naloxone blockade of amphetamine reward in place preference conditioning. *Society for Neuroscience Abstracts*, 11:1173, 1985.
6 Belluzzi, J.D. and Stein, L. Operant conditioning of hippocampal neurons: Role of dopamine D_2 receptors. *Society for Neuroscience Abstracts* 12:706, 1986.
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